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Characterization of Microbial Communities from Pristine and Chlorinated-Ethene-Contaminated Landfill Groundwater

by

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DISCLAIMER

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Characterization of Microbial Communities From Pristine and Chlorinated-Ethene-Contaminated Landfill Groundwater.

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ABSTRACT

Molecular, phospholipid fatty acid analysis (PLFA), and substrate utilization (BIOLOG) techniques were used to assess structural and functional differences between microbial communities from a chlorinated-ethene (CE)-contaminated groundwater at a sanitary landfill. The information will be used to evaluate natural attenuation of the associated CE plume. Two groundwater-monitoring wells were tested. Well LFW 43B was upgradient from the CE contamination. Well LFW 62D was in an area of CE contamination and ongoing bioremediation. Prokaryotic cells in the water from each well were collected on 0.2 micrometer filters, DNA was extracted from the filters and libraries were prepared. For well LFW 43B, 26 clones were examined by sequencing and restriction endonuclease patterns, and all were found to be closely related to *Pseudomonas gessardii* and *P. libaniensis*. For well LFW 62D, 40 bacterial clones were examined, and 17 ribotypes were found, which included representatives of type I and II methylotrophs, *Pseudomonas* spp., *Zoogloea* spp., and other proteobacteria. In an archaeal library from well LFW 62D, all 15 of the clones examined were nearly identical and possessed about 89 %·sequence similarity to *Cenarchaeum symbiosum*. PLFA analysis revealed that the communities from contaminated groundwater contained primarily gram negative bacteria, as indicated by the predominance of the biomarker 16:1w7c and were in the stationary growth phase as indicated by the abundance of cyclopropyl fatty acids cy17:0 and cy19:0 and their respective precursors 16:1w7c and 18:1w7c. Further, PLFA ratios for 16:1w7t/16:1w7c and 18:1w7t/18:1w7c were greater than 0.1, which was indicative of increased cellular membrane permeability. Using BIOLOG GN plates, a similar number of substrates were utilized (>0.1 OD590nm) in LFW43B (max=72) and LFW 62D (max=63) communities, even though inoculum densities were 2-orders of magnitude greater in LFW 62D. The combination of non-selective characterization techniques was useful to further our understanding of the affect of CE-contamination on microbial community structure and metabolic roles.

INTRODUCTION

The Non-Radioactive Waste Disposal Facility (NRWDF) located at the Savannah River Site in Aiken, SC, received sanitary waste, construction material, as well as rags soaked with perchloroethylene (PCE) and trichloroethylene (TCE) for over 20 years. Quarterly groundwater monitoring (starting in 1984) of NRDWF leachate indicated not only the presence of PCE and TCE, but also several microbial transformation products including cis-dichloroethylene (cDCE) and vinyl chloride (VC); however, no other chlorinated ethenes were known to have been disposed of at the NRWDF. Groundwater monitoring data provides convincing evidence that monitored natural attenuation (MNA) of chlorinated ethenes has occurred via reductive dechlorination, including detection of VC down gradient from the initial location of the PCE/TCE plume.

We report here the microbial characterization of groundwater from two distinct monitoring wells at the NRWDF. We provide both molecular, PLFA, and metabolic evidence to support the fact that microbial diversity was higher in the CE-contaminated well as compared to the non contaminated well. Integrated investigations of specific site parameters and microbial community dynamics are needed as a basis for environmental restoration efforts. We hypothesize that the landfill leachate provides multiple substrates to enhance the microbial diversity of the groundwater, as we observed at the contaminated well LFW 62D.

MATERIALS AND METHODS

Determination of groundwater microbial biomass and geochemistry. Groundwater was obtained from two permanent groundwater-monitoring wells within the NRWDF located at the Department of Energy Savannah River Site (Aiken, South Carolina, USA) (Figure 1). Samples were collected on March 16 and 18, 1999, from monitoring well LFW43B, located in a pristine area with no history of contamination, and from monitoring well LFW62D, located within a VC solvent plume one-half mile down gradient from LFW 43B. Approximately six well volumes (70L) were purged prior to

collecting 15-L samples in autoclaved polycarbonate bottles. Groundwater parameters were measured in the field. Samples were stored at 4°C prior to analysis. Aliquots (100 mL) of the groundwater were used for microbiological analysis.

Molecular analysis. A 10-liter aliquot of groundwater was sequentially passed through 2.7, 1.0, 0.7, and 0.2 μm pore-size cellulose filters (Whatman, England). The 0.2 μm filters contained the highest density of cells (>100 cells/field). Therefore, these filters were utilized in subsequent analyses.

PCR and Cloning. The DNA from each filter was subjected to two PCR amplification reactions. The PCR inserts in the resulting clones were sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia (Athens, GA, USA).

Phylogenetic Analysis. A FastA search was performed on all sequences to determine which sequences in the GenBank and EMBL databases were most closely related. All sequences were compared to each other, and if duplicate sequences occurred (>99.6% sequence similarity), then only one of each type was used for further analysis. The program Check Chimera, from the Ribosomal Database Project was used to determine if any of these sequences were chimeric. Phylip 3.5 was used to construct phylogenetic trees on all remaining sequences. The Jukes Cantor formula was used to calculate distances, and the Neighbor joining, Parsimony, and Fitch-Margoliash algorithms were used to examine phylogenetic relationships.

Phospholipid Analysis. Two 3-L aliquots of each groundwater sample were vacuum filtered at the same time as the molecular samples. A 3-liter aliquot of groundwater was sequentially passed through 2.7, 1.0, 0.7, and 0.2 μm pore-size cellulose filters (Whatman). All the filters were immediately packed in ethanol-rinsed aluminum foil and frozen at -70° C. The filters were shipped on ice to Microbial Insights, Inc. (Rockford, TN, USA) for PLFA analysis.

BIOLOG Analysis. Aliquots (150 µl) of groundwater samples were directly inoculated into triplicate BIOLOG GN plates (BIOLOG, Inc., Hayward, CA, USA) and incubated at room temperature for up to 6 days. Triplicate control plates were inoculated with autoclaved deionized water. At 0, 1, 2, 3, 4, 5, and 6 days post-inoculation, absorbance (590 nm) was measured using an automated plate reader (Biotek Instruments, Inc., Winooski, VT). Average color well development (AWCD) was determined as described by Garland (1996).

RESULTS

Microbiology. Table 1 summarizes the results for total culturable bacteria (1% PTYG plates), total bacteria densities (AODC), methanotrophic bacteria by DFA, and geochemical parameters found in the pristine (LFW43B) and contaminated (LFW62D) groundwater. All microbiological tests revealed higher microbial densities in the contaminated groundwater from LFW62D as compared to LFW 43B.

Geochemistry. The groundwater from the contaminated well had higher concentrations for every geochemical parameter measured compared to the non-contaminated groundwater, with the exception of dissolved oxygen (DO) and nitrate (Table 1). Further, elevated chloride levels (up to 37 ppm) were consistent with the dehalogenation of chlorinated solvents.

Molecular.

LFW43B. Approximately 130 colonies containing PCR-amplified bacterial 16S rDNA were obtained from the sample from LFW43B. Eight of these clones were sequenced, and they were virtually identical. Four of the sequences had exactly the same sequence, and four contained one or two different substitutions out of 550 positions examined. This level of substitutions is within the range expected from Taq DNA polymerase error. Since the sequences were greater than 99.6% similar to each other, only one was chosen for subsequent analyses (SRS43BBA18). Plasmids representing 18 more clones from

LFW 43B were digested with *Eco* R1, and the clones were found to contain a DNA banding pattern identical to that of the sequenced clones. Therefore, all 26 clones examined from LFW 43B examined by either sequencing or restriction endonuclease pattern appeared to be from closely related organisms (Table 2).

The sequence of clone SRS43BBA18 was 99.5% similar to that of *Pseudomonas gessardii*. Phylogenetic analysis placed these sequences and *Pseudomonas libaniensis* in the *Pseudomonas* group of the gamma proteobacteria (Figure 2). *P. gessardii* and *P. libaniensis* were first isolated from spring water from France and Lebanon, respectively.

LFW 62D. Approximately 150 colonies containing PCR-amplified bacterial 16S rDNA were obtained from the sample from LFW62D, and forty-one of these clones were sequenced. One sequence was found to be chimeric and was eliminated from further analysis. When the remaining sequences were compared to each other, seventeen ribotypes were found (Table 2). Sequences SRS62DBA43, -11, -07, -03, -19, -09, -21, and -05 were related to the beta proteobacteria, and sequences SRS62DBA39, -44, -37, -10, -32, -12, -24, and -01 were related to the gamma proteobacteria. Sequence SRS62DBA50 was of special interest because, while it was not closely related to any cultured organisms, it was related to 4 uncultured and unclassified eubacterial clones found in a hydrocarbon- and chlorinated-solvent-contaminated aquifer at 87-90% sequence similarity. Sequence SRS62DBA50 had less than 75% sequence similarity to the sequences of any cultured organism in the database.

To determine the most closely related sequences, the clones from the gamma and beta proteobacterial groups and clone SRS62DBA50 were analyzed separately. Within the gamma proteobacteria (Figure 3), SRS62DBA01, -24, -12, -32, -10, and -37 grouped consistently with the Methylococcaceae family. In 93% of the replicates SRS62DBA1, -24 grouped with a cluster of *Methylomonas* species.

SRS62DBA12, -32, -10, and -37 always grouped together with *Methylobacter psychrophilus* and isolate BB5.1, but the branching order within this clade varied (Figure 4). Clones SRS62DBA39 and -44 consistently clustered with the *Pseudomonas* species. Although the branching order within these two clades differed sometimes, SRS62DBA39 was always found clustered with *P. lundensis*, *P. fluorescens*, *P. brassicacearum* and *P.*

syringae, and SRS62DBA44 was always found grouped with *P. libaniensis* and *P. gessardii.*

Within the beta-proteobacteria (Figure 5), SRS62DBA21 and -05 clustered with the methylotrophs in the beta proteobacteria group 85% of the time; otherwise it grouped with the *Thiobacillus* group. SRS62DBA43, -11, -07 and -03 clustered consistently with *Pseudomonas mephitica* and *Janthinobacterium lividum.* However, the branching order of SRS62DBA07 and -03 varied, which caused some low bootstrap values within this clade. SRS62DBA19 and -09 clustered with *Pseudomonas lemoignei*, but the branching order within this group changed. Similar results were also obtained by parsimony and the Fitch-Margoliash analyses. In these analyses, SRS62DBA09 and –19 remained associated with the clade including *Herbaspirillum*, *Janthinobacterium*, and *Duganella.* However, -09 and –19 did not always associate with *P. lemoignei* and branched deeper within the clade instead. Moreover, within the clade that contained SRS62DBA03 and -07 the branching order varied.

The 500 base pair sequence that resulted from clone SRS62DBA50 possessed only low similarity to other sequences in the Genbank and EMBL databases, and 798 more positions of this clone were sequenced. A phylogenetic tree was constructed with the almost complete 16S rDNA sequence of SRS62DBA50 and sequences from a diverse collection of cultured prokaryotes. SRS63DBA50 did not group consistently with any group of organisms. The program Check Chimera indicated that the SRS62DBA50 sequence was not chimeric, but other analyses were done to support this conclusion. FastA analyses were performed on several different portions of the sequence (300 base pairs) and each portion had relatively the same % sequence similarity to the same sequences in the Genbank and EMBL databases. Since the sequence folded into a recognizable 16S rRNA molecule and did not possess small regions with high sequence similarity to other sequences, SRS62DBA50 did not appear to be chimeric.

Approximately 160 colonies containing PCR-amplified archaeal 16S rDNA were found in the archaeal library from LFW 62D. Fifteen clones were sequenced (Table 2). Sequences of 10 clones had 100% sequence similarity and the remaining 5 each contained 1 independent substitution out of 450 positions. Only one clone (SRS62DAR03) was chosen for further analysis. This clone was fully sequenced (1346

nucleotides) for the phylogenetic analysis. Within the GenBank and EMBL databases, the sequence with the highest similarity (89%) to that of SRS62DAR03 was the crenarchaeote clone *Cenarchaeum symbiosum* from marine sponges (Figure 6). This assignment was robust and detected in phylogenetic trees constructed using neighbor-joining, Fitch-Margoliash, and parsimony analyses.

Phospholipid fatty acid analysis. Phospholipids were directly extracted from groundwater microbial biomass captured on membrane filters, without prior plating or culturing on a selective-enrichment medium. As such, no attempt was made to identify individual bacteria comprising the groundwater communities. In the pristine groundwater (LFW 43B), only four different phospholipids were detected: 18:1w7c, 14:0, 16:0, and 18:0. The distribution was uneven and primarily composed of 18:1w7c (66%), which is common in many pseudomonads. In contrast to the relatively limited pristine groundwater community, the contaminated groundwater (LFW62D) contained 17 different phospholipids. Monoenoic lipids composed 77% of the PLFA profile and are common in Gram-negative bacteria or proteobacteria. The monoenoic PLFA 16:1w8c accounted for 6.5% and was found in *Methylomonas* spp. Other monoenoic PLFAs included 18:1w8c at 5.3%, common in *Methylosinus*; 10me16:0 at 1.1%, common in *Desulfobacter*; and i17:1w7C at 0.4%, common in *Desulfovibrio*. Terminally branched saturated PLFAs at 2.0%, common in Gram-positive or sulfate-reducing bacteria were also detected.

The Gram negative communities in LFW62D contained detectable biomarkers for growth phase (Figure 9) and environmental stress (Figure 10). Although clones related to Methylomonas were common in the library from LFW62D, clones related to *Methylosinus* , sulfate-reducing, or Gram-positive bacteria were not found. However, since these PLFA biomarkers represented a low percentage of all the phospholipids present, it is possible these microbes were indeed present in the groundwater samples, but that their 16S rDNA was not detected because of their low abundance. Alternatively, it is possible that these PLFA markers are not unique but instead are present in one of the groups of the organisms detected by 16S rDNA sequencing. The Gram negative communities in LFW62D contained detectable biomarkers for growth phase (Figure 9).

and environmental stress (Figure 10). In contrast LFW43B indicated no detectable turnover rate or signs of environmental stress. The physiological status of Gram-negative communities can be assessed from ratios of different monoenoic biomarkers. Specifically, 16:1ω7c and 18:1ω7c are converted to cyclopropyl fatty acids (cy17:0 and cy19:0) as microbes move from a log to a stationary phase of growth (i.e. slowing of growth).

BIOLOG. Substrate utilization was defined as a substrate well with an optical density (OD_{590nm}) greater than 0.1 after subtraction of the control well (containing only redox dye groundwater, and no substrate). Inoculum density was not adjusted and was 2-orders of magnitude greater in contaminated vs pristine wells. Microbial communities from pristine groundwater utilized 72 substrates and from contaminated groundwater used 62 substrates. Microbial communities from both pristine and contaminated groundwater exhibited similar lag phases that occurred at 0-2 days following inoculation. The rate was 0.07 (at 2-5 days) in the pristine groundwater and 0.19 (at 2-5 days) in the contaminated groundwater. Maximum AWCD values (0.6) were reached at 6 days for pristine groundwater and 5 days for contaminated groundwater.

DISCUSSION

All microbiological tests revealed higher microbial densities in the contaminated compared to pristine groundwater. Total cell densities and culturable heterotrophs were approximately 2-orders of magnitude greater in contaminated vs. pristine wells. This was expected, since labile carbon sources are thought to move with the CE plume. However, it was surprising the number of methanotrophs in pristine groundwater (2.2×10^2 [is this number right?] cells/mL; Table 1) because that area of the landfill is severely oligotrophic and historical documents indicate that no known waste had been disposed of there

The molecular and PLFA results on the relative diversity of the two wells is strong For example, as mentioned the monoenoic biomarker 16:1ω8c accounted for 6.5% of the phospholipids and is found in *Methylomonas*. Similarly, 7.5% of the bacterial clones sequenced from LFW62D grouped with *Methylomonas*. With the exception of SRS62DBA50, all of the bacterial clones sequenced from LFW62D grouped with the proteobacteria, which also concurred with the PLFA results .

Thirty percent of the bacterial clones sequenced from LFW62D clustered with the *Methylophilus* and *Methylobacillus* members of the β proteobacteria. *Methylophilus* and *Methylobacillus* are methanol- oxidizers. Methanol is one of the byproducts of methane oxidation, and it is likely that these bacteria are utilizing the methanol produced by the type I methanotrophs. Methanol oxidizers are commonly found in association with methane-oxidizing bacteria for this reason.

The pseudomonas and burkholderia groups contain metabolically diverse bacteria. Some pseudomonas and burkholderia, such as *P. putida* and *B. cepacia*, can oxidize chlorinated ethenes. Results here demonstrate that 35% and 5% of the bacterial clones sequenced grouped with the burkholderia and pseudomonas groups, respectively. With the exception of a close relative of *P. gessardii* these bacteria were not detected in the uncontaminated well (LFW 43B), and it is likely that the contaminating compounds have enriched for these organisms and act as a source for their carbon and energy.

This study provides significant insight regarding characteristics associated with microbial communities in contaminated aquifers. The aquifers at the Savannah River Site offer many unique environments, including contaminated and pristine, that could host different microbial communities. Data obtained from 16S rRNA, BIOLOG, and PLFA profiles provides evidence that the microbial populations in aquifers can vary based on site geochemical parameters. Bioremediation is known to be more successful where there is indigenous diverse population of microorganisms capable of mineralizing a wide range of substrates. At the SRS, microbial characterization has proven to be of critical importance in determining the successful outcome of field-scale bioremediation deployments. Future microbial analyses at this site will help determine whether and how subsurface microbial communities change as contaminate plumes advance through them.

In addition, this information can lead to management practices of these indigenous microbial communities to maintain the diversity and activity needed to achieve long term environmental restoration goals.

Acknowledgements

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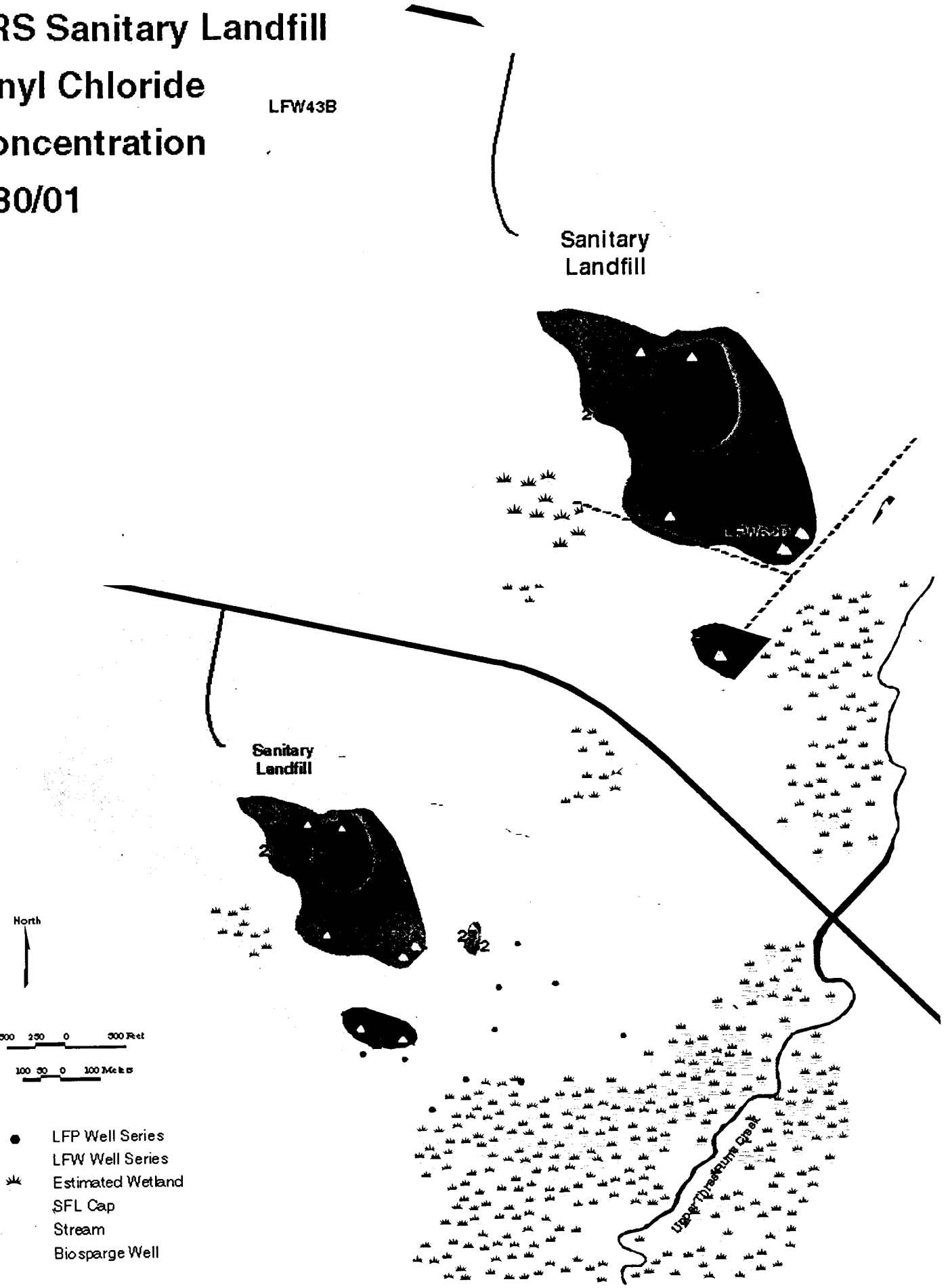
Figure 1. Map of Landfill.

SRS Sanitary Landfill
Vinyl Chloride
Concentration
9/30/01



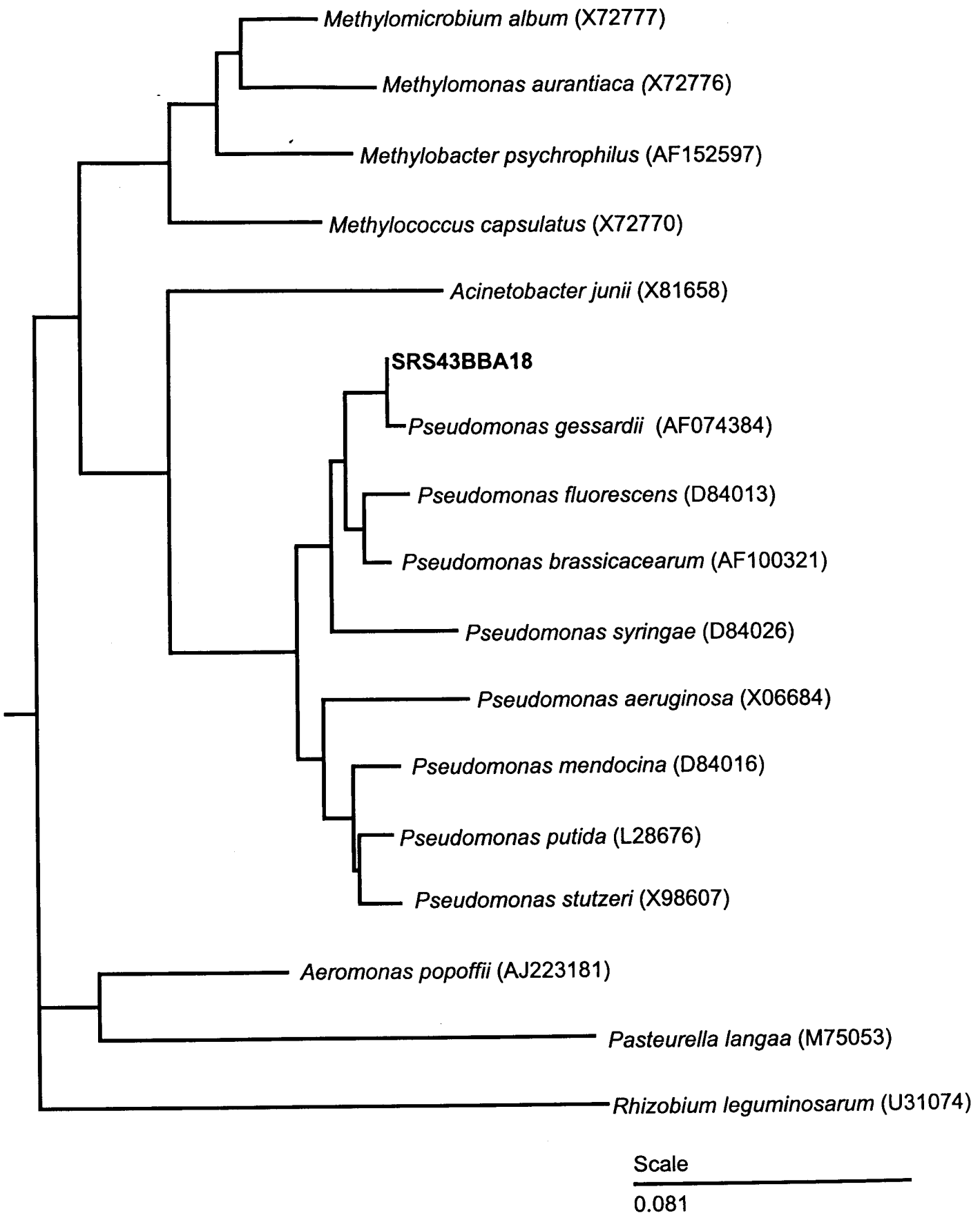


Figure 2. Phylogenetic tree generated by the neighbor-joining method from an allignment of 559 nucleotide positions. The clones from LFW 43B were related to the γ-Proteobacteria. The scale bar indicat indicates the accession number for the sequence.

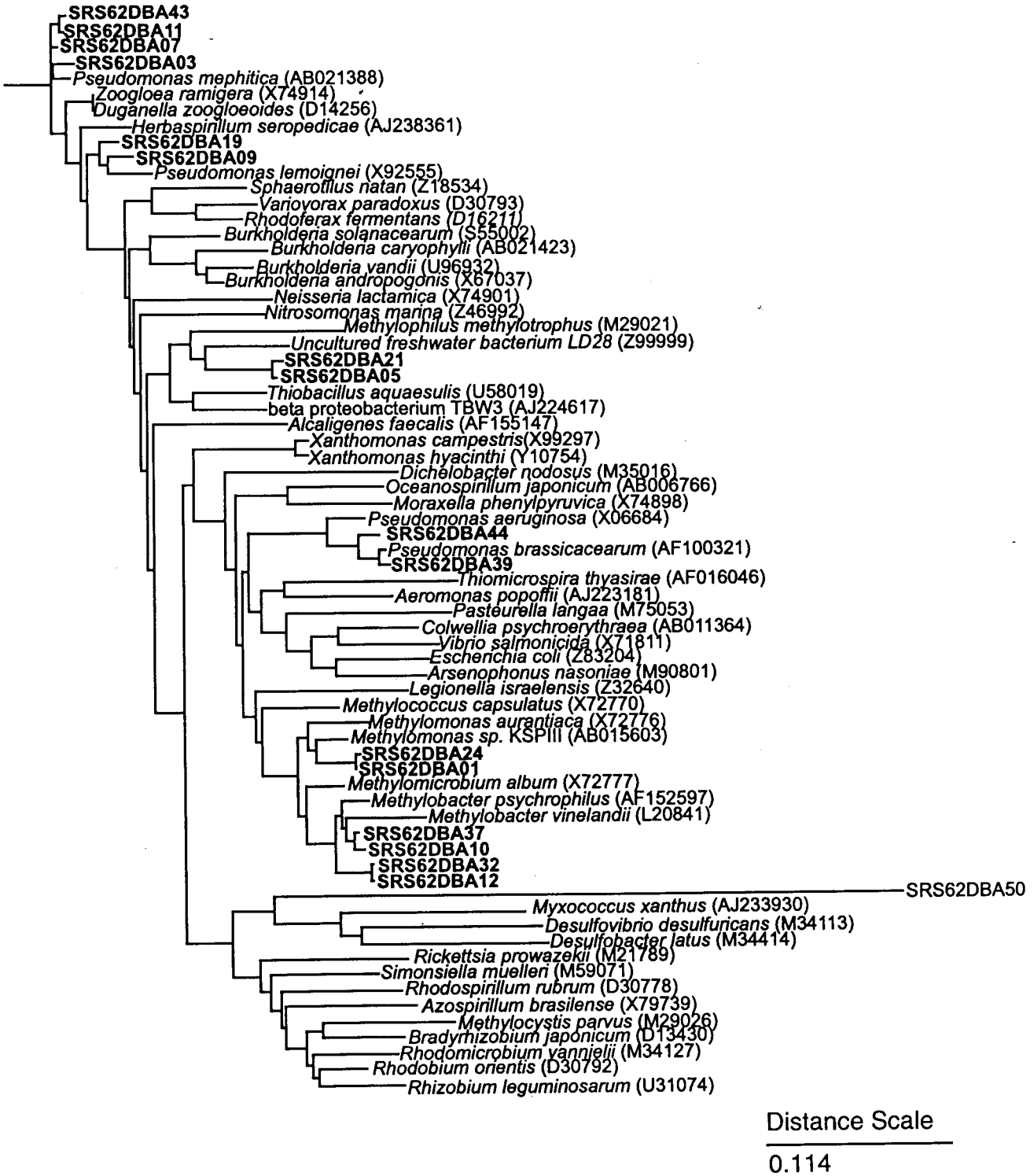


Figure 3. Phylogenetic tree generated by the neighbor-joining method from an allignment of 490 nucleotide positions, showing the relationships between LFW 62D clones and clones related to the α,β,γ,δ-Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence.

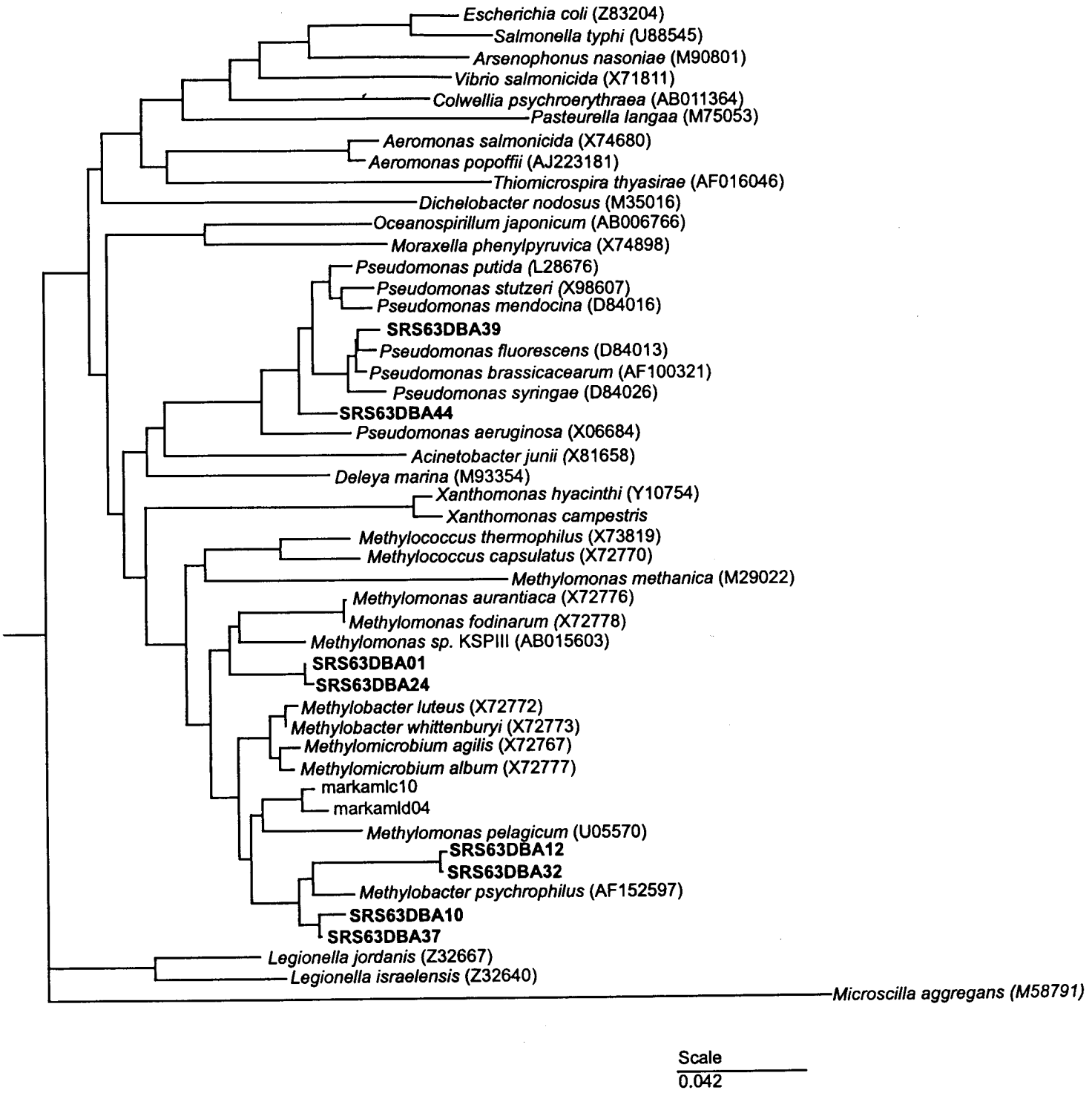


Figure 4. Phylogenetic tree generated by the neighbor-joining method from an allignment of 502 nucleotide positions, showing the relationships between LFW 62D clones and clones related to the γ-Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence. markamlc10 and markamld04 refer to two novel type I methanotrophic isolates obtained from a landfill by Mark Wise in 1998 (unpublished data)

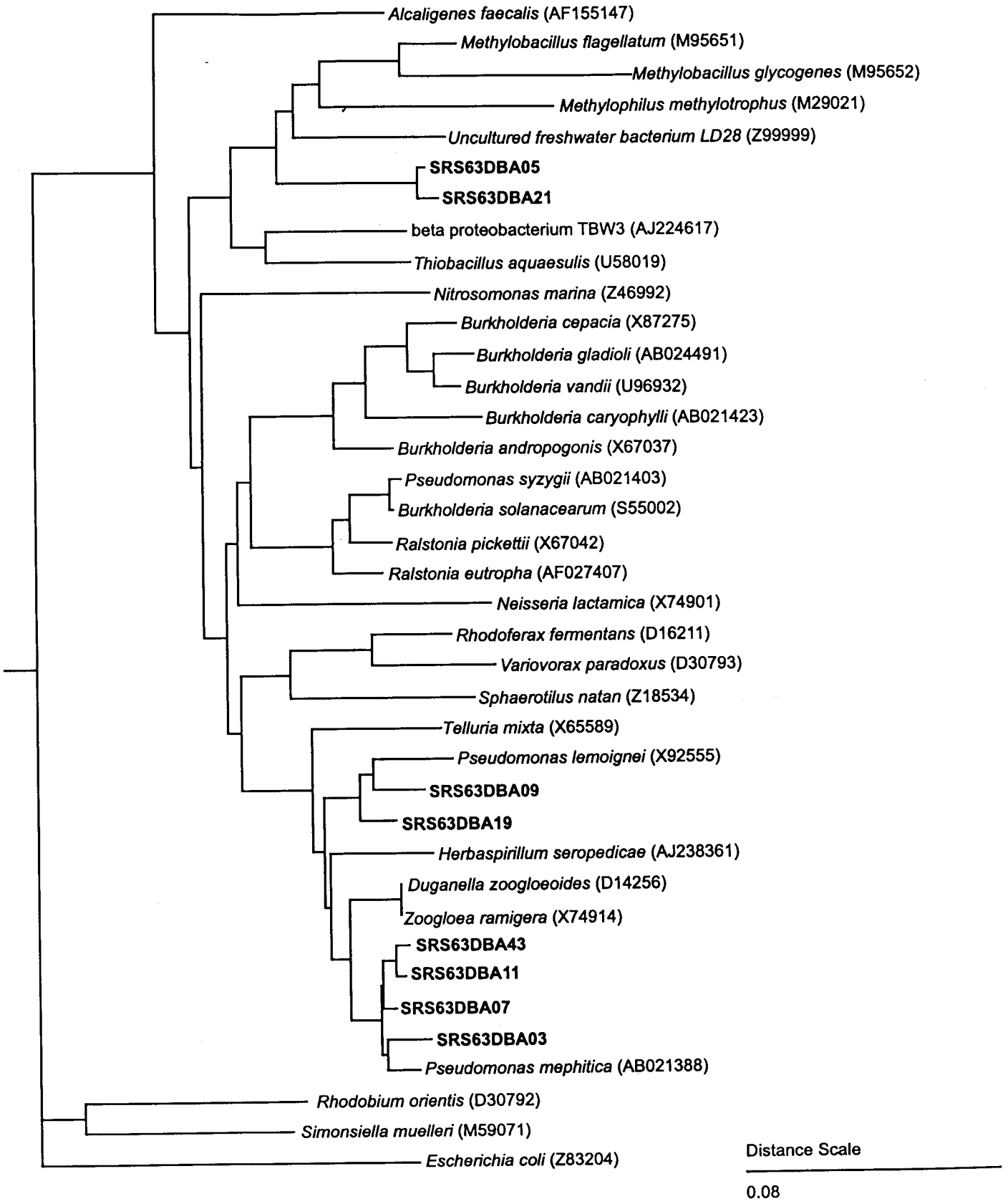


Figure 5. Phylogenetic tree generated by the neighbor-joining method from an allignment of 469 nucleotide positions, showing the relationships between LFW 62D clones and clones related to the β-Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence.

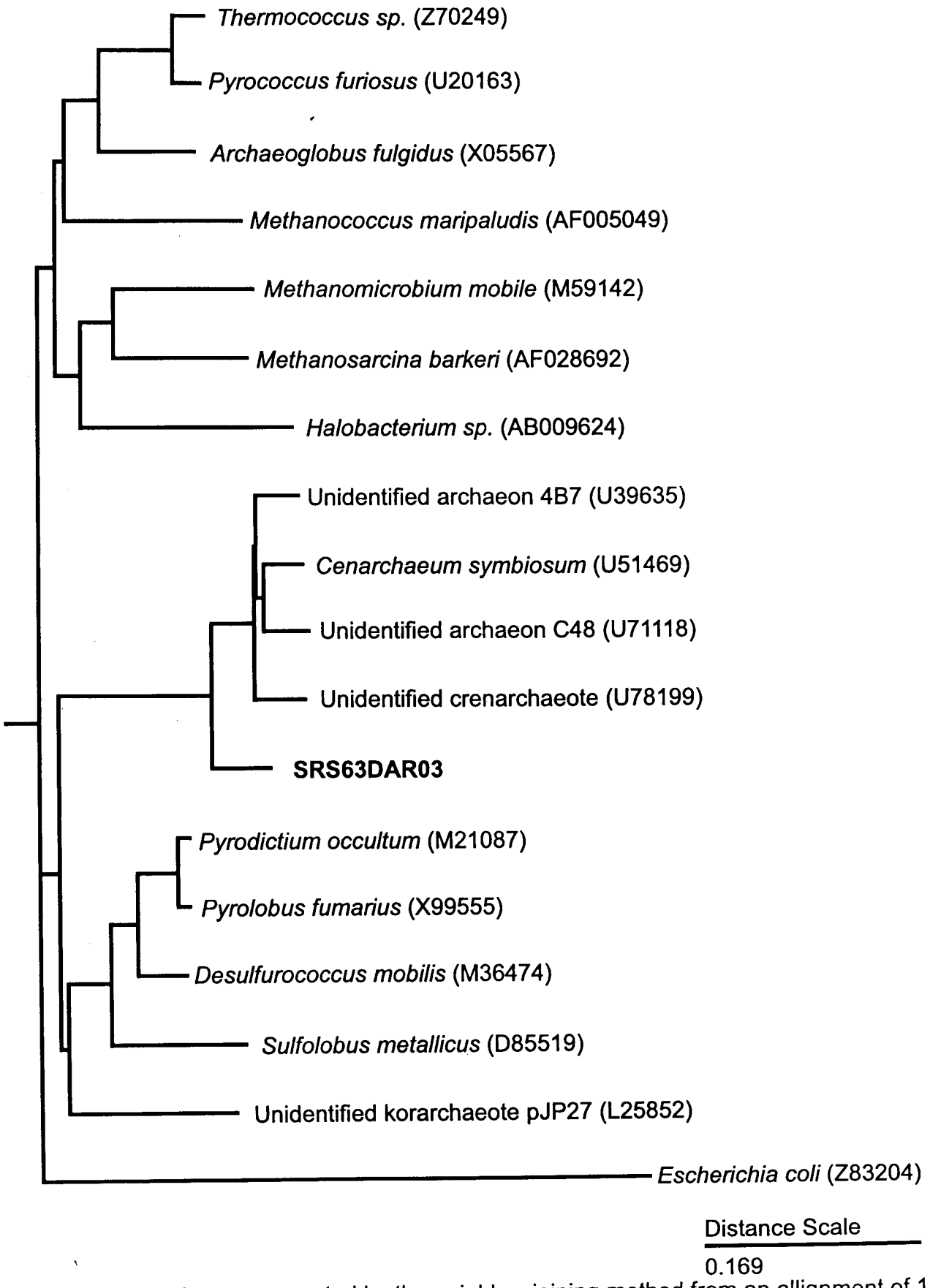


Figure 6. Phylogenetic tree generated by the neighbor-joining method from an allignment of 1,339 nucleotide positions, showing the relationships between LFW 62D archaeal clones and clones related to the organisms in the Archaea domain. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence.

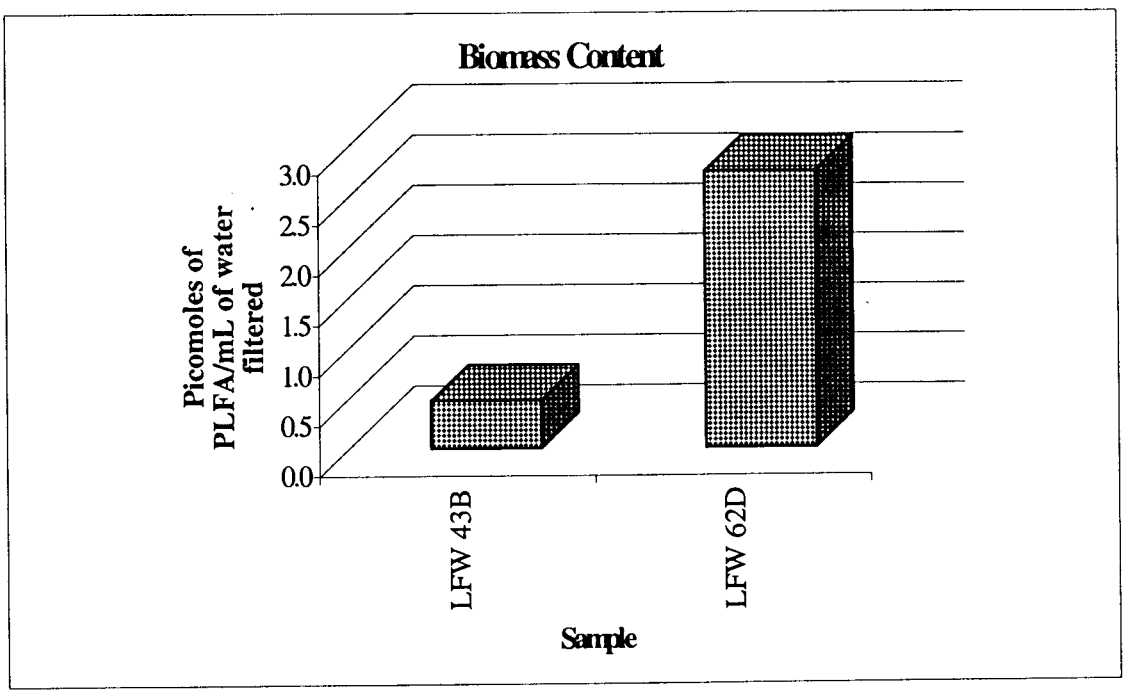


FIG. 7. Groundwater biomass content.

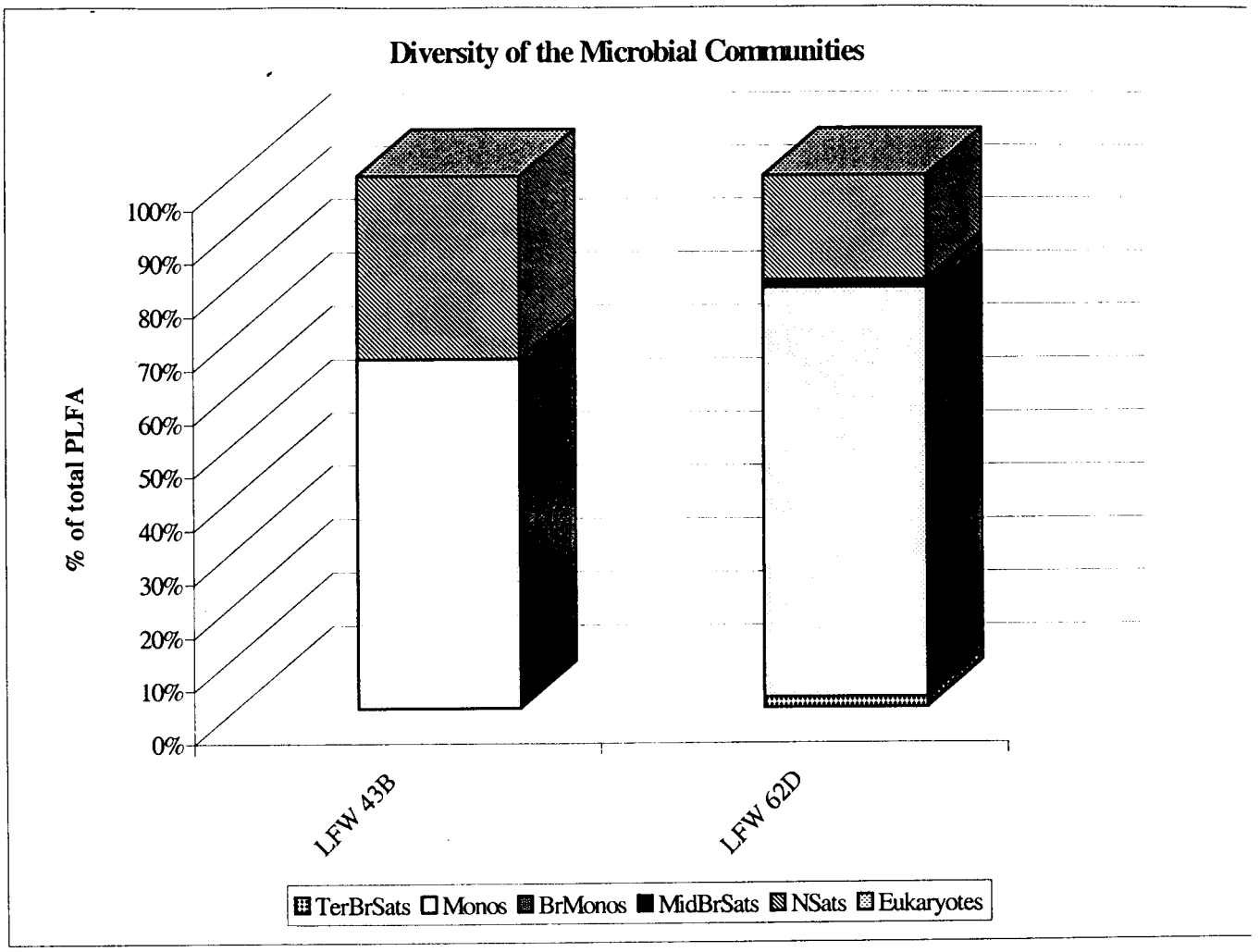


FIG. 8. Percentage of total PLFA detected in the samples.

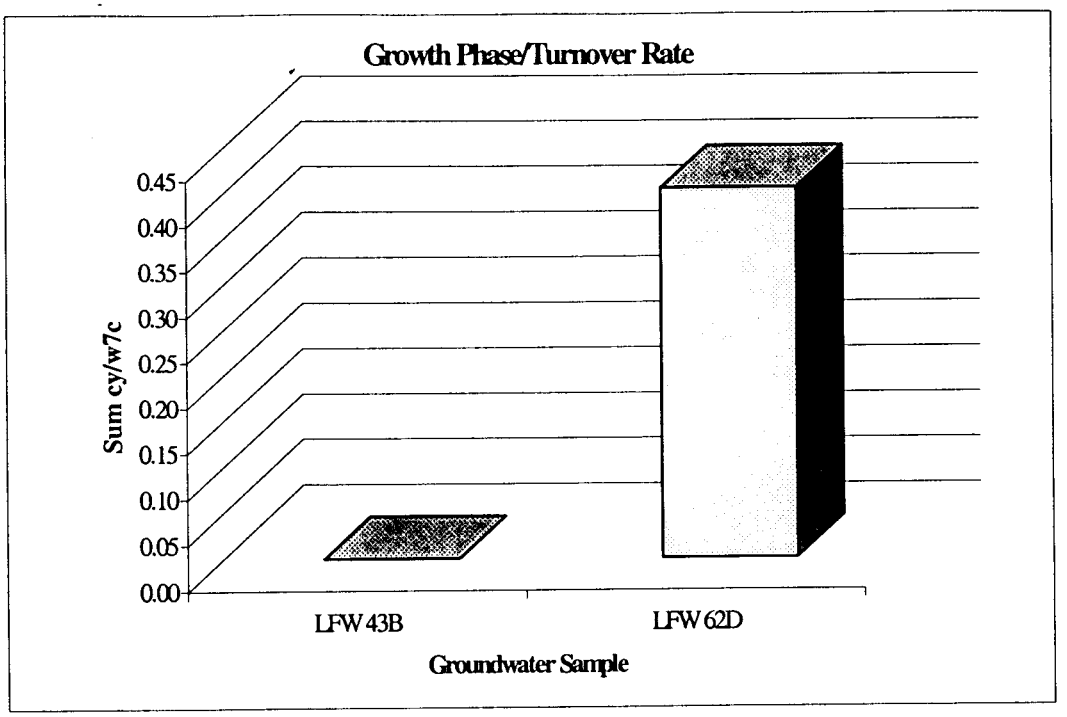


FIG 9. Growth phase of the Gram negative communities.

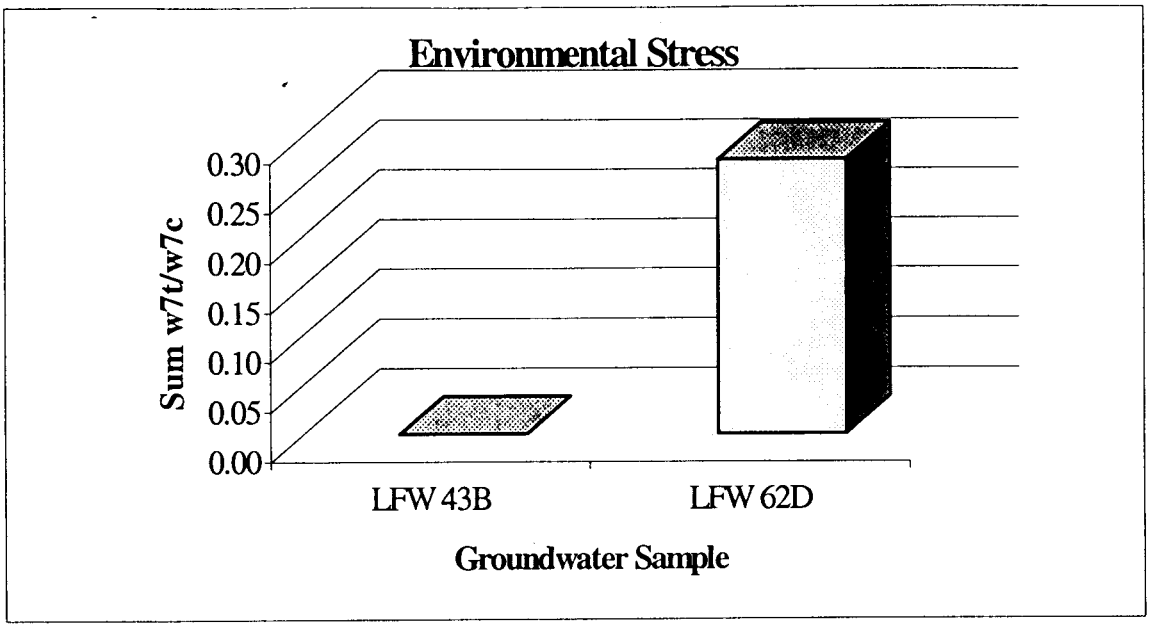


FIG 10. Membrane permeability in the Gram negative communities.

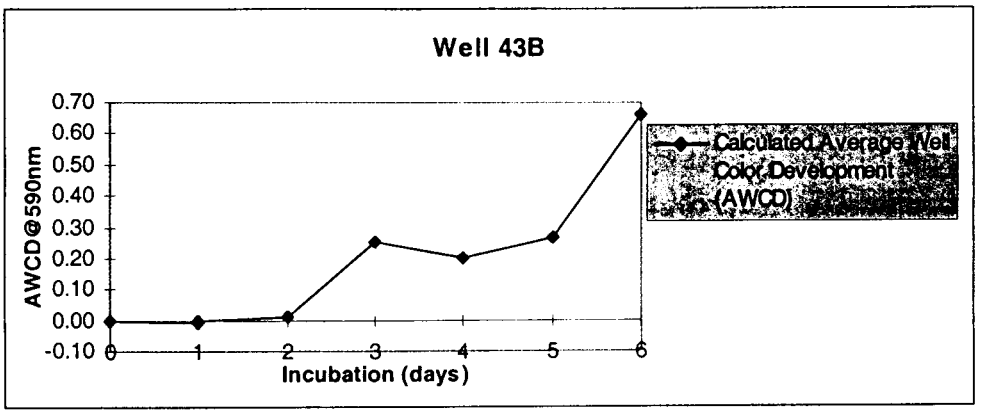


FIG11a. AWCD over time in non-contaminated groundwater (LFW 43B).

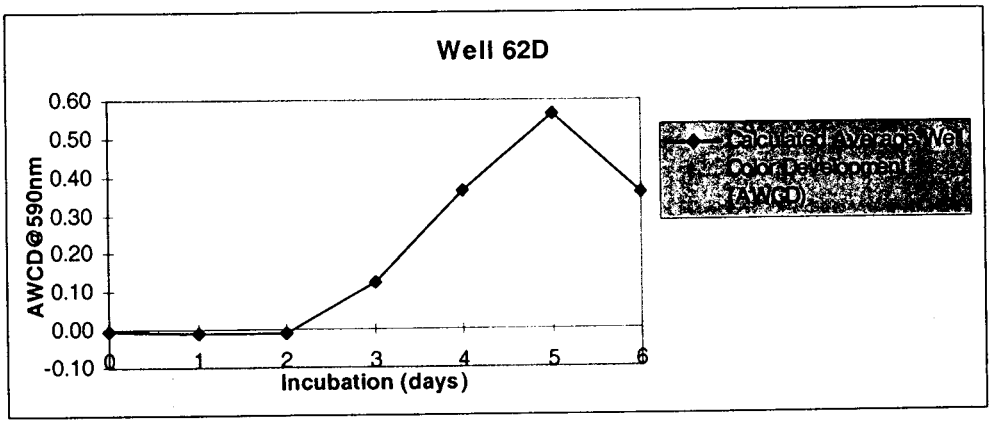


FIG11b. AWCD over time in contaminated groundwater (LFW 62D).

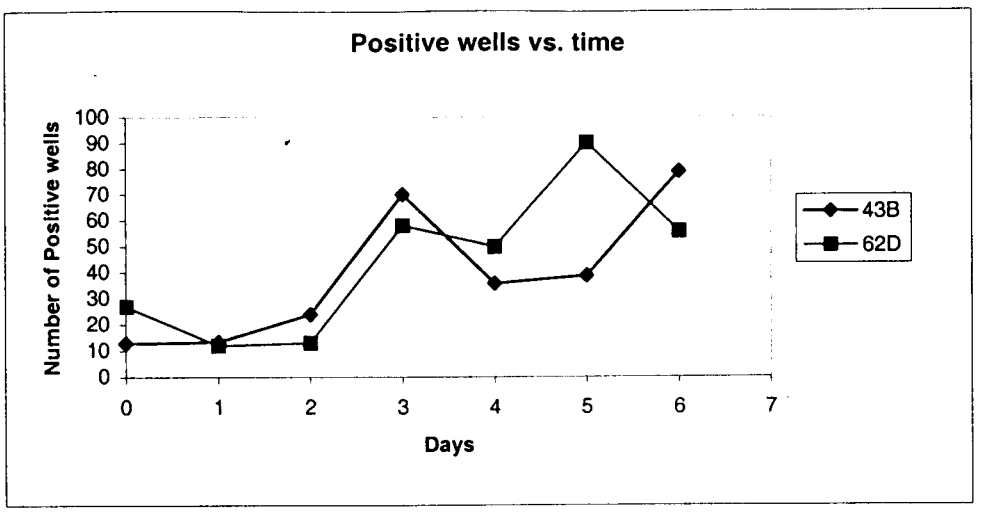


FIG 11. Number of individual BIOLOG substrates utilized (>0.1 OD_{590nm}) over time.

TABLE 1. Summary of microbiological and geochemical analysis on groundwater samples from wells LFW 43B and LFW 62D.

[could add location as a row to the table, ie. "contaminated, pristine"

Parameter	Unit	LFW 43B Pristine	LFW 62D Contaminated
Plate Counts	cfu/ml	1.05E+02	8.25E+03
AODC	Cells/ml	1.32E+04	2.27E+06
DFA's	Cells/ml	2.24E+01	1.63E+02
TCE	(ug/l)	0	10
1,1 DCE	(ug/l)	0	70
PCE	(ug/l)	0	6
VC	(ug/l)	0	26
CB	(ug/l)	0	22
Methane	(ppmv in 10 ml sample)	15	11085
Total Iron	UGL	27.4	1780
Chloride	(mg/l)	1.9	37
Nitrate	(mg/l)	4	<1.0
Sulfate	(mg/l)	1.6	5.8
Temperature	C	18.8	21.7
Conductivity	µS/cm	21	170
Alkalinity	mg/l	1	7
DO	Mg/L	9.82	3.57
Turbidity	NTU	0.3	14.7
Depth	Ft	37.2	21.9

TABLE 2. 16S rDNA sequences from LFW 62D and 43B amplified with bacterial primers and archaeal primers.

Representative Clones	Number of clones found with this ribotype[a]	Closest Relative[b]	Percent similarity[c]
SRS43BBA18	26	*Pseudomonas gessardii*	99.5
SRS62DBA05	7	beta proteobacterium TBW3	90.8
SRS62DBA21	5	beta proteobacterium TBW3	90.3
SRS62DBA03	1	Zoogloea ramigera	96.8
SRS62DBA07	1	*Zoogloea ramigera*	97.8
SRS62DBA11	1	*Zoogloea ramigera*	97.2
SRS62DBA19	1	*Zoogloea ramigera*	96.2
SRS62DBA43	1	*Pseudomonas mephitica*	98.0
SRS62DBA09	9	*Pseudomonas lemoignei*	94.2
SRS62DBA12	3	*Methylobacter psychrophilus*	97.9
SRS62DBA32	1	*Methylobacter psychrophilus*	97.5
SRS62DBA10	1	*Methylomonas sp.* T20	97.4
SRS62DBA37	3	*Methylomonas sp.* T20	98.2
SRS62DBA01	2	*Methylomonas sp.* KSPIII	96.2
SRS62DBA24	1	*Methylomonas sp.* KSPIII	95.9
SRS62DBA39	1	*Pseudomonas brassicacearum*	98.9
SRS62DBA44	1	*Pseudomonas gessardii*	99.8
SRS62DBA50	1	*Acetobacterium wieringae*	71.6
SRS62DAR03	15	*Cenarchaeum symbiosum*	89.3

[a]Ribotype includes all sequences with ≥99.6% sequence similarity.
[b]Refers to the sequence in the Genbank or EMBL databases (Benson *et al.*, 1999; Stoesser *et al.*, 2000) that possessed the highest percent similarity.
[c]Percent similarity = 100 X (number of identical bases) / (total number of positions compared)